SERINE/THREONINE PROTEIN PHOSPHATASE 5 INTERACTS WITH THE HUMAN CHECKPOINT PROTEIN RAD17 AND REGULATES ATM KINASE ACTIVITY

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The identification of genetic alterations that contribute to breast cancer is critical for effective treatment and prevention of the disease. A hallmark characteristic of cancer cells is abrogation of cell cycle checkpoints that control response to DNA damage. These checkpoints are regulated by certain kinases including ATM (ataxia telangiectasia mutated). Through a yeast two-hybrid screen, we have uncovered a physical association between Rad17 (an ATM-regulated protein) and a serine-threonine phosphatase, PP5. Interestingly, a reduction in PP5 expression was found to abolish irradiation-induced ATM kinase activation, suggesting that the Rad17/PP5 complex may play a critical role in the activation of ATM in response to DNA damage. Most importantly, PP5 was found to be able to form a stable complex with endogenous Rad17 in 293T and the breast cancer cell line, MCF-7. Based on the observation of complex formation between Rad17 and PP5, we had originally reasoned that PP5, as a multifunctional phosphatase might play a role as a negative regulator of the checkpoint pathway by countering the kinase activity of ATM on Rad17 phosphorylation. However, our recent studies yielded an unexpected, but highly significant finding that a reduction in PP5 expression resulted in a significant repression of ATM kinase activation in response to IR stimulation. Consistent with the repression of ATM kinase activity under this condition, the phosphorylation of p53 on Ser-15 and Rad17 on Ser-635, known ATM substrates, was also significantly repressed. Another approach to address the role of PP5 was through the generation a dominant negative mutant. A small internal deletion in the C-terminal region of the catalytic domain of PP5 results in a mutant protein that maintains its localization in the nucleus but loses its catalytic activity. We have generated a GST-fusion PP5 protein that contains this deletion and demonstrated that this mutant retains its ability to interact with Rad17. Under an in vitro assaying condition, we confirmed that this mutant PP5 had lost its catalytic activity. When this mutant PP5 was expressed in cells, ATM activation upon damage was repressed. Taken together, our preliminary results strongly suggest that PP5 may regulate ATM kinase activity.

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MEVALONATE PROMOTES THE GROWTH OF HUMAN BREAST CANCER CELLS IN VITRO AND IN NUDE MICE AND INHIBITS EXPRESSION OF THE CYCLIN-DEPENDENT KINASE INHIBITOR $p21^{cip1}$

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Mevalonate synthesis by HMG-CoA reductase is rate-limiting for the production of cholesterol and non-sterol isoprenoids. Independent of cellular requirements for sterols, mevalonate also exerts direct control over cell cycle progression at the G1 to S interphase – it is required for the initiation of DNA synthesis and for cell proliferation. Depletion of mevalonate by the statins, a group of drugs that competitively inhibit HMG-CoA reductase, results in growth arrest of cells in G1. The mevalonate pathway may be a useful target for anti-cancer therapies. Malignant cells are often characterized by increased mevalonate synthesis. However, the mechanism by which mevalonate regulates cell cycle progression is unknown. Furthermore, it is also unknown whether a causal link exists between elevated mevalonate and cancer promotion.

To determine the effects of exogenous mevalonate on the growth of breast tumors, nude mice were inoculated in the mammary fat pad (mfp) with ~10^6 MDA-MB-435 human breast cancer cells. One week later the mice were implanted *sc* with mini-osmotic pumps delivering either 1.92 μmol (0.25 μL)/h mevalonate or 0.25 μL/h isotonic saline (control). By delivering mevalonate directly to the area of the mfp, hepatic clearance of mevalonate was circumvented (data not shown). Tumors from mevalonate treated mice weighed significantly more than those from control mice at 13 weeks (1.52 ± 0.26 vs. 0.81 ± 0.27g, $p<0.05$). Tumor cross-sectional areas were also greater in mevalonate treated mice than in controls (215.0 ± 24.8 vs. 133.1 ± 28.11 mm^2, $p<0.05$). To determine whether the growth promoting effects of mevalonate result from cell cycle alterations, MDA-MB-435 cells were treated in culture with mevalonate for 72 h. Cell proliferation rates and cell cycle distribution were assessed as well as mRNA and protein levels of the cyclin-dependent kinase inhibitor $p21^{cip1}$, an inhibitor of the active cyclin/cyclin-dependent kinase complexes required for G1 progression. Cell proliferation rates were 43.2 ± 8.1% higher in cells treated with mevalonate compared to controls ($p<0.0001$). Mevalonate treated cells had significantly lower levels of $p21^{cip1}$ mRNA and protein compared to controls.

It is clear from our results that mevalonate promotes the growth of human breast cancer cells both *in vivo* and *in vitro*, and that these effects may be due to a reduction in levels of the cell cycle inhibitor $p21^{cip1}$. Cancer cells are characterized by both impaired control of cell cycle progression and enhanced mevalonate synthesis. Our research on the role of mevalonate in regulating the cell cycle is important for developing strategies for the prevention and treatment of breast cancer.

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KINETOCHORES AS TARGETS FOR CHEMOTHERAPY

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The kinetochore is a macromolecular protein complex that provides two critical functions that are essential for accurate chromosome segregation in mitosis. Kinetochores contain proteins that specify the mechanical interactions between chromosomes and the spindle and are thus essential for chromosome motility. Kinetochores also contain checkpoint proteins that monitor interactions between kinetochores and microtubules to ensure that cells with even a single unaligned chromosome will not exit mitosis prematurely. Given that kinetochores function specifically in mitosis, it would appear to be a highly desirable target for development of novel anti-cancer drugs. Toward this end, we have applied recently developed technology of siRNA to selectively deplete from HeLa cells the checkpoint protein, BUB1 and the microtubule motor, CENP-E. HeLa cells depleted of BUB1 are no longer able to arrest in mitosis when its spindle is disrupted by nocodazole. Instead the checkpoint defective cells exit mitosis without dividing to be polyploid cells. Further analysis showed that the loss of BUB1 prevented several other checkpoint proteins, including CDC20, hMAD1, hMAD2 and hROD from assembling onto kinetochores. Thus, BUB1 appears to specify the assembly of a subdomain of the kinetochore that is critical for checkpoint functions. In contrast to the BUB1 studies, depletion of CENP-E from HeLa cells induces a prolonged mitotic arrest as chromosomes fail to properly align.

We have expanded our studies to include the breast cancer cell lines MCF-7 and MDA-486. Preliminary date indicate that we can deplete BUB1 and CENP-E from these cell lines using siRNA. Interestingly, the response may differ slightly between Hela cells and MDA-468 cells. Depletion of CENP-E from MDA-MB-486 cells result in fewer mitotically blocked cells as was found for Hela cells. This indicates that MDA-MB-468 cells may not have an intact spindle checkpoint. Assessment of the response of these cells to simultaneous inhibition of microtubule formation and checkpoint proteins may reveal enhanced cell killing. Ongoing studies may reveal that inhibition of spindle checkpoint pathway may sensitize cells to microtubule inhibitors such as vinblastine and paclitaxal.

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THE S-PHASE CHECKPOINT COUPLES DNA REPLICATION WITH MITOSIS BY CONTROLLING THE APC SPECIFICITY FACTOR CDC20

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Progression of breast cells to the malignant state involves the acquisition of genome instability. Recent studies determined that mutations in S-phase checkpoint factors were those most likely to cause genome instability [Myung et al. 2001a,b]. The S-phase checkpoint prevents mitosis while DNA replication is ongoing. Our studies aim to determine the mechanism of S-phase checkpoint control, an important goal if we are to understand cancer etiology. In the clinic, checkpoint integrity of tumor cells is highly relevant for designing treatment regimes, since compromised checkpoint controls affect the sensitivity of cells to therapeutic agents.

Since checkpoint factors are conserved between humans and lower eukaryotes, we use yeast to rapidly analyze checkpoint pathways. Our experiments use green fluorescent protein-tagged mitotic spindles and chromosome loci to determine cell cycle position in checkpoint mutants.

We determined that budding yeast prevent mitosis during S-phase by regulating the Anaphase Promoting Complex (APC) specificity factor, Cdc20. Specifically, Mec1 (a yeast homolog of human ATM, mutated in Ataxia telangiectasia patients) represses the accumulation of Cdc20 protein in S-phase, an event needed for activation of mitotic APC. The human CHK2 homolog, Rad53, is also needed for this checkpoint response, but initiation of mitosis in *rad53* mutants does not require APC components other than Cdc20. Further, genetic data indicate that Mec1 and Rad53 have overlapping but distinct roles in S-phase checkpoint signaling.

These results link the budding yeast ATM and CHK2 homologs to the regulation of APC activity. However, APC activity is not absolutely required for the initiation of mitosis. Therefore, the inappropriate S-phase activity of Cdc20, responsible for inducing mitosis, is at least partially independent of the APC. These studies have defined part of the complex mechanism that prevents cell division when genome duplication is incomplete, and therefore protects against genome instability and malignant progression.

Myung et al. *Cell*, 2001. 104(3):397-408; Myung, et al. *Nature*, 2001. 411(6841):1073-6.

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A NOVEL, CELL CYCLE-DEPENDENT CDK SUBSTRATE THAT REGULATES CENTROSOME DUPLICATION IN HUMAN CELLS

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It has recently been shown that centrosome duplication and separation are tightly linked to certain cell cycle events. In particular, activation of certain cyclin-dependent kinases (CDKs) coincides with initiation of duplication. However, relatively few CDK targets involved in initiating these events have been identified. Here, we describe a biochemical screen for CDK substrates that we used to isolate and characterize a novel target, CP110 that is phosphorylated by CDKs in vitro and in vivo. Human CP110 localizes strictly to centrosomes. Its expression is strongly induced in late G1 phase of the cell cycle, coincident with the initiation of centrosome duplication. We have ablated CP110 with RNAi, and these experiments indicate an essential role for this protein in centrosome duplication. Remarkably, CP110 plays a role in the regulation of ploidy, as disruption of CP110 phosphorylation provokes dramatic polyploidy. In total, these data strongly suggest that CP110 is a physiological target of cyclin dependent kinases that regulates centrosome duplication. De-regulation of CP110 may contribute to genomic instability, and potential links with human cancer are being investigated.

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INDOLE-3-CARBINOL REGULATION OF CYCLIN-DEPENDENT KINASE 2 (CDK2) ENZYMATIC ACTIVITY THROUGH ALTERATIONS IN THE SUBCELLULAR LOCALIZATION OF CDK2/CYCLIN E

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Indole-3-carbinol (I3C), a naturally occurring component of Brasssica vegetables, such as broccoli, is a promising anti-cancer agent that we have previously shown to induce a G1 cell cycle arrest of human breast cancer cell lines (MCF-7 and MDA-MB-231) independent of estrogen receptor signaling. Concurrent with the growth arrest, I3C drastically decreases CDK2 kinase activity without changes in protein levels. In exploring the mechanisms by which I3C regulates CDK2 kinase activity, we show that the decrease in CDK2 enzymatic activity is not due to changes in the positive phosphorylation event at the threonine 160 position or by inhibitory phosphorylation event at the tyrosine 15 position. We also investigated the level of CDK inhibitors (p21, p27) that are known to associate with the CDK2 complex and found that the level of p21 associated with the CDK2 complex remain unaltered with I3C treatment. Subcellular localization studies of CDK2 and cyclin E using indirect immunofluorescence show that cyclin E and CDK2 redistributes from the nucleus to the cytoplasm with I3C treatment. This is further supported by western blot analysis of cyclin E and CDK2 protein levels in cytoplasmic and nuclear fractions of treated and untreated cells. I3C treated cell lysates contain elevated levels of both CDK2 and cylin E in the cytoplasmic fractions as compared to control. These findings indicate that I3C may be affecting interactions between CDK2 and cyclin E or other cellular proteins that causes the decreased levels of CDK2 in the nucleus, either by decreasing the import of the CDK2 complex or increasing the rate of export. Additionally, we have shown that the redistribution of cyclinE/CDK2 is specific to the I3C response since breast cancer cells that have been treated with Tamoxifen, an antiestrogen that also decreases CDK2 enzymatic activity and has been shown by our lab to synergize in suppressing growth and CDK2 kinase activity of MCF-7 breast cancer cells, does not change the redistribution of the cyclin E/CDK2 complex into the cytoplasm. This suggests that simply decreasing CDK2 kinase activity is not sufficient to cause the change in the subcellular localization of cyclin E/CDK2 complex.

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CONTROL OF CELL CYCLE TRANSITIONS BY THE SCF UBIQUITIN LIGASE

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Progression through the cell division cycle is controlled by cyclin dependent kinases and their inhibitors (CKIs). The levels of both cyclins and CKIs are tightly controlled through multiple mechanisms, including transcriptional and post-transcriptional pathways. One such pathway for controlling levels of cyclins and CKIs is ubiquitin-mediated proteolysis. Our previous genetic and biochemical studies have elucidated the SCF ubiquitin ligase pathway, which uses F-box proteins as substrate specific adaptors to link phosphorylated ubiquitination substrates with a core ubiquitin ligase containing Skp1, Cul1 and Rbx1. We have identified more than 70 F-box proteins in the mouse and human genome. Recent analysis of members of this family have identified Fbw7 as a specificity factor for degradation of cyclin E. Cyclin E is overexpressed in breast cancer and is thought to be a prognostic marker for breast cancer. Fbw7 is structurally related to Cdc4, an F-box protein in budding yeast that is responsible for degradation of the Sic1 CKI. Using RNAi in Drosophila cells and siRNA in HeLa cells, we have found that ablation of Fbw7 leads to stabilization of cyclin E, consistent with a role for Fbw7 in cyclin E turnover in vivo. Consistent with this, cyclin E is ubiquitinated by SCFFbw7 complexes in vitro. We have identified several phosphorylation sites in cyclin E from human cells, including S89, S372, T380, and S384. Interaction of cyclin E with Fbw7 requires T380 but other phosphorylation events in the C-terminus are not required for association in vitro. Modification of T380 is catalyzed by GSK3-beta but not by Cdk2 or Cdc2 in vitro. However, inactivation of GSK3/shaggy by RNAi in Drosophila does not stabilize cyclin E, suggesting the involvement of additional kinases in cyclin E control. Additional efforts seek to develop general methods to identify ubiquitination substrates involved in cell cycle control and breast cancer.

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P16 LOSS IS NOT REQUIRED TO IMMORTALIZE HUMAN MAMMARY EPITHELIAL CELLS

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While human fibroblasts exhibit a limited number of divisions before entering senescence or M1, human mammary epithelial cells (HMECs) exhibit a different pattern. Previous reports have shown that primary cultures of HMECs undergo a self-selection or M0 stage after an initial growth period (approximately 10-15 population doublings). Emergence from this transient growth plateau is correlated with loss of p16 expression. It has been reported that both loss of p16 function and reactivation of telomerase activity (hTERT expression) are required for immortalization of HMECs. We have previously shown that the premature growth arrest (M0) and loss of p16 in epithelial cells are due to inadequate growth conditions that can be overcome by the use of feeder layers.

We sought to determine whether HMECs, under the proper growth conditions, could be immortalized with human telomerase without the need to abrogate p16. We first transferred the early passage HMECs (derived from organoid cultures) from plastic culture dishes to dishes with feeder layers (mitomycin-treated 3T3 cells). These hTERT-expressing cells bypass M0 and immortalize. Transferring these cells back to plastic culture dishes or infecting the cells with Ras induced an increase in p16 protein expression, indicating that p16 was not lost or abrogated.

These findings support the hypothesis for a stress-induced abrogation of p16 of HMECs growing on plastic culture dishes, but more importantly indicate that loss of p16 is not required for HMEC immortalization. Inactivation of p16 is clearly occurring in many tumors, and it may be silenced because it contributes to a checkpoint arrest of invasive cells that migrate into an inappropriate environment. However, to date there is no evidence supporting a role for p16 in the regulation of replicative aging. In the absence of in vivo data suggesting other counting mechanisms, we propose the adoption of the functional definition that culture conditions are adequate if they permit cells to reach a telomere-based replicative senescence that can be bypassed by the expression of telomerase.

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CONSTITUTIVE OVEREXPRESSION OF CYCLIN D1, BUT NOT CYCLIN E, CONFERS ACUTE RESISTANCE TO ANTIESTROGENS IN T-47D BREAST CANCER CELLS

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Cyclin D1 and cyclin E are overexpressed in approximately 45% and 30% of breast cancers, respectively, and adverse associations with patient outcome have been reported. The potential roles of cyclin D1 and cyclin E expression as markers of therapeutic responsiveness to the pure steroidal antiestrogen Faslodex (ICI 182780) were investigated using T-47D breast cancer cell lines constitutively overexpressing cyclin D1 or cyclin E. Measurement of S phase fraction, phosphorylation states of the retinoblastoma protein, pRb, and cyclin E-Cdk2 kinase activity demonstrated that overexpression of cyclin D1 decreased sensitivity to antiestrogen inhibition at 24 and 48 hours. Overexpression of cyclin E produced a less pronounced early cell cycle effect indicating only partial resistance to antiestrogen inhibition in the short-term. In ICI 182780-treated cyclin D1-overexpressing cells, sufficient Cdk activity was retained to allow pRb phosphorylation and cell proliferation, despite an increase in the association of p21 and p27 with cyclin D1-Cdk4/6 and cyclin E-Cdk2 complexes. In long term colony-forming assays, antiestrogen treatment inhibited colony growth in cyclin D1- or cyclin E-overexpressing breast cancer cells, but with a ~2-2.5 fold decrease in sensitivity. This was associated with a reduction in cyclin D1 levels and a decline in cyclin E-Cdk2 activity in cyclin D1-overexpressing cells, and the maintenance of cyclin E-p27 association in the cyclin E-overexpressing cells. These data confirm that cyclin D1 expression and cyclin E-p27 association play an important role in antiestrogen action and suggest that cyclin D1 or cyclin E overexpression has subtle effects on antiestrogen sensitivity. Further studies to identify the mechanism leading to downregulation of cyclin D1 following long-term antiestrogen treatment and to assess the relationship between antiestrogen sensitivity and expression of cyclin D1, cyclin E or p27 in a clinical setting are required.

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THE SPINDLE ASSEMBLY CHECKPOINT IN FISSION YEAST

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In order to prevent the gain or loss of genetic information during mitosis, which may cause cell death or predispose cells to cancer, all sister chromatids must be attached to and then separated by the mitotic spindle. The spindle assembly checkpoint protects the integrity of the genome by initiating a cell cycle delay if all chromatids are not properly attached to the spindle. Defects in checkpoint function occur in human cancer cell lines and in human breast cancers, suggesting that the checkpoint normally prevents genetic changes that accompany cancer development.

Most human spindle checkpoint proteins were identified by their similarity to checkpoint proteins first identified genetically in yeast. In the fission yeast Schizosaccharomyces pombe, loss of the checkpoint genes mph1 or mad2 renders cells sensitive to spindle disruption. Because these cells fail to arrest in mitosis for spindle repair, they undergo a lethal mitosis. Overexpression of mph1 in wild type cells activates the spindle checkpoint, arrests cells in mitosis and is therefore toxic. Because mph1 acts before the non-essential protein mad2 in the checkpoint pathway, the toxic effect of mph1 overexpression can be suppressed by deletion of the mad2 gene.

Genetic evidence indicates that all of the components of the spindle checkpoint pathway have not yet been identified. A genetic screen to isolate additional suppressors of mph1 overexpression has yielded a temperature sensitive mutant called 4101, which is defective in an apparently novel component of the checkpoint pathway. As expected for a checkpoint mutant, 4101 cells fail to arrest the cell cycle in response to spindle disruption. However, 4101 cells also have mitotic defects and lose viability at the restrictive temperature, even in the absence of spindle disruption. The identification and characterization of the gene mutated in strain 4101 will contribute to an elucidation of the mechanism of spindle checkpoint function and its role in the prevention of genomic instability.

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ANALYSIS OF CYCLIN D1 FUNCTIONS IN VIVO

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Inactivation of the retinoblastoma protein (pRb) is one of the most frequent events in cancer. This can be achieved through direct mutation of the gene encoding pRb, but is more often the consequence of hyperactivation of regulatory cyclin D/cdk complexes. Examples of this include overexpression of cyclin D1 in breast tumors, elevated activity of cdk4 in glioblastoma and melanoma, and loss of $p16^{INK4}$ in many tumors. Indeed tumor types that frequently overexpress cyclin D1 show infrequent pRB loss, suggesting that cyclin D1 overexpression provides an oncogenic advantage that extends beyond loss of pRB. This might derive from tissue-specific functions of cyclin D1. For example, cyclin D1 has been reported to activate transcription in complex with the estrogen receptor, directly regulate the transcription factors DMP-1, v-myb, and STAT-3, and, with cdks, to act as a "sink" for p21 and p27, inhibitory proteins that prevent the activity of cyclin E/cdk2. Moreover, it has been proposed based on experiments in cells lacking p21 and p27 and in mice expressing cyclin E in place of cyclin D1 that cyclin D1's kinase activity is dispensable for proliferation. Because of these observations, it is now unclear if kinase activation by cyclin D1 underlies its role in tumorigenesis, and any efforts to inhibit cyclin D1-dependent tumors specifically are compromised by a lack of certainty as to the biochemical role of cyclin D1 in dysregulated proliferation. To address this, we have produced alleles of cyclin D1 that retain catalytic activity but lack the LXCXE "pRb binding" domain or bind cdk4/6 without ability to activate the kinase. This latter mutant, K112E, cannot reverse pRb-mediated proliferation arrest, but does profoundly interfere with expression of senescence markers, suggesting retention of a noncatalytic function capable of interrupting aspects of pRb-induced cell cycle exit. We can now move these analyses in vivo, exploiting the fact that mice lacking cyclin D1 are viable but show profound defects in breast and retinal development and are less prone to experimental induction of cancer. To achieve this, we have produced mice expressing the K112E allele from the endogenous cyclin D1 locus, and will shortly have LXCXE mutant mice as well. The K112E knockin mice exhibit phenotypes similar to the knockout. We are currently investigating the biochemical properties of the K112E protein *in vivo*. We will also commence an assessment of the K112E protein's ability to mediate neu induced tumorigenesis.

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HEC1, HINT1, AND ZW10 SEQUENTIALLY FORM A COMPLEX AT KINETOCHORE RESPONSIBLE FOR CHROMOSOME SEGREGATION

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Hec1, an evolutionarily conserved coiled-coil protein, plays important roles in multiple mitotic events. Hec1 protein is expressed in a cell cycle dependent manner, most abundantly in the S and M phases. It localizes to nuclei and a portion of them distributes to centromeres during M phase. It has been shown that Hec1 interacts with MSS1 and modulates the proteolysis of mitotic cyclins. Moreover, Hec1 regulates chromosome segregation partially through SMC proteins.

To further explore the mechanisms of how Hec1 executes its function in chromosome segregation, we have identified an interacting protein, Hint1, which in turn also binds to ZW10 at kinetochore. By immunostaining and co-immunoprecipitation, these three proteins are co-localized at kinetochore and form a complex specifically at M phase. These results suggest that Hec1 recruits Hint1 and ZW10 sequentially to kinetochores. Since Hec1 is overexpressed in most of cancer cells and inactivation of Hec1 leads to lethality of cells, this complex will serve as a useful target for developing strategy to treat cancer cells.

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ASSOCIATION BETWEEN P21WAF1/CIP1 AND DNA POLYMERASE DELTA

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P21 is known to inhibit cell cycle progression by binding to G1 cyclin-dependent kinase (CDK) complexes. p21 also binds to proliferating cell nuclear antigen (PCNA) and inhibits the ability of PCNA to activate DNA polymerase delta (pol delta), the principal replicative DNA polymerase that is involved in both DNA replication and DNA repair.
Using a yeast two hybrid screening technique and the p50 subunit of DNA polymerase delta as a bait, we found that p21 binds to the polymerase delta p50 subunit. We confirmed the p21 and p50 interaction by using GST-p50 and GST-p21 pull-down assays. We also obtained in vivo evidence for this interaction through co-immunoprecipitation of p21 and p50 in MCF7 breast cancer cells by using monoclonal antibody against p50 or polyclonal antibody against p21. We found that p21 associates with other components of the polymerase delta complex by using GST-p21 pull down and by Far-Western techniques. A pairwise yeast two hybrid assay showed that the N-terminal 1-107 amino acid residues of p50 and the C-terminal 83-164 amino acid residues of p21 may be involved in this interaction. We conclude that p21 can bind to DNA polymerase delta directly. The functions of the interaction between DNA polymerase delta and p21 are still under investigation.

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PKB PHOSPHORYLATES P27, IMPAIRS NUCLEAR IMPORT OF P27, AND MEDIATES TGF-BETA RESISTANCE IN HUMAN CANCERS

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Transforming growth factor-beta (TGF-beta) inhibits cell proliferation by inducing G1 phase cell cycle arrest. Resistance to G1 arrest by TGF-beta often accompanies malignant tumor progression so that cancer cells not only can escape this normal growth control mechanism but also acquire proliferation and survival advantage by secreting TGF-beta to inhibit normal and, especially, immune cell proliferation. We provide evidence that oncogenic activation of the PI3K/PKB pathway may oppose TGF-beta mediated G1 arrest through phosphorylation of the cdk inhibitor, p27kip1. PI3K/PKB activation was increased in TGF-beta resistant lines and downregulation of PI3K/PKB activity restored G1 arrest by TGF-beta. Moreover, transfection of constitutively activated PKB (PKBDD) conferred TGF-beta resistance, increased the abundance of p27-cyclin D1-cdk4 complexes and caused cytoplasmic accumulation of p27. PKB/Akt was shown to phosphorylate p27 at threonine 157 and p27 mutation converting threonine 157 to alanine abolished this effect. While fluorescently tagged wild type p27 accumulated in the nucleus of TGF-beta sensitive cells, it was expressed in both the nucleus and cytoplasm of PKBDD transfected cells. However, the p27T157A localized almost exclusively to the nucleus despite constitutive expression and activity of PKB in PKBDD transfectants. Cytoplasmic p27 was seen in 52/128 primary human breast cancers in association with PKB activation. For all levels of nuclear p27 staining, cancers with cytoplasmic p27 showed poorer differentiation and patient outcome. In summary, we demonstrated PKB activation contributes to TGF-beta resistance and tumor progression by impairing nuclear import and action of the cdk inhibitor p27. An understanding of this mechanism may be useful in making diagnostic and therapeutic decisions for breast cancer patients.

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AP-1 BLOCKADE INHIBITS BREAST CANCER GROWTH BY INDUCING A G1 CELL CYCLE BLOCK

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The AP-1 transcription factor is a complex of proteins composed of Jun, Fos, Jun dimerization partners (JDP1 and JDP2) and the closely related activating transcription factors (ATF/CREB) subfamilies. AP-1 is a central component of signal transduction pathways in many cells, though the exact role of AP-1 in controlling cell growth and transformation of epithelial cells is unknown. We have previously shown that AP-1 complexes are activated by peptide and steroid growth factors in both normal and malignant breast cells, and that blocking AP-1 by over-expressing a dominant negative form of cJun (cJun-DN, Tam67) inhibits breast cancer cell growth both *in vivo* and *in vitro*. We hypothesize that Tam67 inhibits cell growth by altering the expression of cell cycle regulatory proteins, thus causing a cell cycle block. In the present study, we used clones of MCF7 breast cancer cells that express Tam67 under the control of an inducible promoter. First, we determined the effect of AP-1 blockade on cell growth using a cell proliferation assay and on apoptosis using TUNEL and PARP cleavage assays. We then performed 3H-thymidine incorporation and flow cytometry assays to investigate whether Tam67 affects the cell cycle. We observed that Tam67 inhibited cell growth and caused a block in the G1 phase of the cell cycle. Tam67 also caused apoptosis in the absence of serum, but did not induce apoptosis in the presence of serum. Next, we did western blotting and CDK Kinase assays to determine the effect of Tam67 on Rb phosphorylation, on the expression of cell cycle regulatory proteins, and on CDK activity. We demonstrated that Tam67 inhibited Rb phosphorylation and reduced E2F activity. We also found that Tam67 decreased the expression of the D and E cyclins, reduced CDK2 and CDK4 activity, and increased the expression of the CDK inhibitor P27. These results demonstrate that Tam67 inhibits breast cancer growth by inducing inhibitors of cyclin dependent kinases (such as P27) and by reducing the expression of cyclins involved in transitioning from G1 into S phase of the cell cycle. These molecular changes effectively inhibit breast cell proliferation. These studies will lay the foundation for future attempt to develop new agents for the prevention or treatment of breast cancer.

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STUDY OF CELLULAR FACTORS THAT ARE IMPORTANT FOR GENE AMPLIFICATION

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Breast Cancer progression depends upon the accumulation of many genetic alternations among which DNA amplification seems to play a prevalent role. Recent studies show that 80-90 % of breast carcinomas are associated with gene amplification. Unfortunately, no effort has been put so far to study the mechanism of amplification. Each amplicon most likely has an origin of replication. In Drosophila, DmORC (Origin Recognition Complex) binds to specific DNA sequences at chorion locus and this binding is essential for chorion gene amplification. In this study, we characterized human ORC by biochemical and genetic approaches.

In an attempt to purify recombinant human ORC from insect cells infected with baculoviruses expressing HsORC subunits, we found that human ORC2, -3, -4, and -5 form a core complex, suggesting that the interaction of ORC1 and -6 subunits with the core ORC2-5 complex is extremely labile. We found that the C-terminal region of ORC2 interacts directly with the N-terminal region of ORC3. The C-terminal region of ORC3 was, however, necessary to bring ORC4 and ORC5 into the core complex. A fragment containing the N-terminal 200 residues of ORC3 (ORC3N) competitively inhibited the ORC2-ORC3 interaction. Overexpression of this fragment in cancer cells blocked the cells in G1, providing the first evidence that a mammalian ORC subunit is important for the G1-S transition in mammalian cells.

To investigate the function of ORC in human cells, we created a hypomorphic mutation in the ORC2 gene of a cancer cell line through homologous recombination. This mutation reduced ORC2 protein levels by 90%. The G1 phase of the cell cycle was prolonged, but there was no effect on the utilization of either the c-Myc and b-globin cellular origins of replication. However, we found that cells carrying this mutation failed to support the replication of an extrachromosomal plasmid bearing the oriP replicator of Epstein Barr virus (EBV), and this defect was rescued by reintroduction of ORC2. Geminin, an inhibitor of the mammalian replication initiation complex, inhibited replication of the oriP plasmid. The result concurrently identifies a novel means by which to cure cancer with gene amplification where the amplicons are carried as episomes.

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ROLE OF THE CELL-CYCLE REGULATORY MOLECULES p27 AND CYCLIN D1 IN THE PROLIFERATION OF MAMMARY EPITHELIAL CELLS

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Increased expression of cyclin D1 and decreased expression of the dual function cyclin-dependent kinase (CDK) inhibitor/assembly factor p27 occur commonly in human breast cancer and are associated with a poor prognosis. To investigate the roles of these two modulators of cell cycle progression in normal mammary epithelial cell proliferation and differentiation, mammary gland development was studied in p27-/- and cyclin D1-/- mice. Since female p27-/- mice are infertile, development of p27-/- mammary glands during pregnancy was studied in mammary epithelium transplanted into Rag 1-/- hosts. Although there were no differences in gross morphology of the mammary gland transplants, significant increases in DNA synthesis were observed in p27-/- compared to p27+/+ mammary glands at 6 and 14 days of pregnancy. Western blotting of cyclin D1 immunoprecipitates from p27+/+ and p27-/- mammary epithelial cell (MEC) cultures showed that, although p27 is an assembly factor for association between D-type cyclins and CDKs, lack of p27 did not prevent formation of cyclin D1-Cdk4 complexes. During the first 1-2 days of culture cyclin E-Cdk2 activity was higher in p27-/- MEC than p27+/+ MEC. These data suggest a role for p27 in inhibiting cyclin E-Cdk2 in mammary epithelium but contrast with the published observations of impaired mammary development and proliferation in p27-/- mammary epithelium. Further investigation of the role of p27 in this tissue will be necessary to identify mechanisms potentially underlying the adverse effects of reduced p27 in breast cancer. Female cyclin D1-/- mice do not lactate due to a defect in lobuloalveolar development. Consistent with this observation, ductal branching was equivalent in the mammary glands of cyclin D1+/+ and cyclin D1-/- animals throughout pregnancy but reduced alveolar bud formation was observed in cyclin D1-/- animals. The initial peak of mammary epithelial DNA synthesis at days 3 to 5 of pregnancy was of similar timing and magnitude in cyclin D1-/- and wildtype animals. However, from days 8-16 DNA synthesis was significantly reduced in cyclin D1-/- mammary epithelium. Similarly, in estrogen and progesterone-treated animals mammary epithelial DNA synthesis was transient in cyclin D1-/- animals but maintained over at least 6 days in cyclin D1+/+ animals. These data suggest an inability to sustain continuous proliferation rather than an overall slower rate of proliferation in cyclin D1-/- mammary epithelium. Identification of the mechanism for this defect may provide insight into the necessity for cyclin D1 function in the actions of some mammary oncogenes.

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ROLE OF MITOTIC EVENTS IN TAXOL-MEDIATED APOPTOSIS IN BREAST CANCER

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A commonly used approach to the management of locally advanced breast cancer involves the sequential use of chemotherapy, surgery, followed by radiation and adjuvant chemotherapy. With the advent of paclitaxel, there is increasing interest in the use of the drug in the neoadjuvant setting. Our data show that treatment of xenograft tumors in mice with paclitaxel leads to cell cycle arrest in M-phase that is accompanied by phosphorylation of Bcl-2 and other mitotic epitopes. Further, in many epithelial tumor cells lines, the paclitaxel-induced mitotic arrest is transient. Our laboratory and others have demonstrated that these biochemical events are associated with decreased proliferation and increased apoptosis in the presence of paclitaxel, and may serve as molecular markers of paclitaxel chemoresponsiveness in human tumors. We hypothesize that patients with locally advanced breast cancer who show the greatest degree of M-phase arrest, Bcl-2 and Cdc25C phosphorylation after paclitaxel treatment will be the ones who have the highest rate of complete pathologic response. Moreover, we postulate that we can find additional predictive markers of paclitaxel response by examining protein profiles of pre- and post-paclitaxel exposed tissue using the power of mass spectrometry. These interrelated hypotheses are currently being tested through the following specific aims. Specific Aim 1: To predict the degree of tumor response from paclitaxel-mediated changes in markers of cell cycle position, proliferation, and apoptosis that result from paclitaxel administration in patients with locally advanced breast cancer. Specific Aim 2: To evaluate protein expression profiles by mass spectrometry in biopsy material collected pre- and post-paclitaxel treatment. In sum, we postulate that there is a molecular profile that can be identified from biopsies collected pre- and post-paclitaxel treatment that will predict which patients will show the greatest degree of response to paclitaxel as measured by the degree of pathologic response at time of definitive surgery. Our mass spectrometry-based analyses of protein profiles from pre- and post-paclitaxel biopsy material is generating data that will allow insight to mechanisms of paclitaxel anti-tumor activity and resistance.

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THE ROLE OF CHK1 IN BREAST EPITHELIAL CELL CHECKPOINT FUNCTION

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Breast cancer continues to be the leading cause of death in American women between 30 and 70 years of age and approximately 1 in 10 women will develop breast cancer during their lifetime. Thus, the following must continue to be a high priority: basic research focused on determining the molecular basis of breast cancer and translation of this information to new treatments that will exploit the molecular defects in breast cancer cells. The genetic evolution of normal breast epithelial cells into cancer cells is largely determined by the fidelity of DNA replication, repair, and division. Cell cycle arrest in response to DNA damage is integral to the maintenance of genomic integrity. The control mechanisms that restrain cell cycle transition after DNA damage are known as cell cycle checkpoints. Recent evidence from our laboratory and others suggest that tumor cells with defective DNA damage cell cycle checkpoint function have increased sensitivity to anticancer agents. Therefore, a further understanding of the biochemical pathways that mediate checkpoint function may lead to the identification of more effective breast cancer treatments. Currently our studies are focused on the G2 cell cycle checkpoint signaling pathway in breast epithelial cells and how one component of this checkpoint pathway, the Chk1 kinase, may offer the potential for therapeutic intervention. Currently we are: (1) determining how Chk1 is regulated after exposure of human breast epithelial cells to anticancer agents, and (2) determining if ablation of Chk1 in mammary epithelial cells abrogates G2 checkpoint function and sensitizes mammary epithelial cells to currently used anticancer agents. Understanding the function of Chk1 will enhance our knowledge of the checkpoint controls involved in preventing transformation of normal breast epithelial cells, and may eventually lead to development of novel therapeutic treatments for breast cancer patients.

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ROLE OF AIB1 IN ESTROGEN-DEPENDENT CYCLIN D1 EXPRESSION

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Blocking estrogen receptor function with antiestrogens is a method currently used to treat and prevent breast cancer. Thus, understanding the downstream signaling mechanisms and genetic changes that lead to estrogen-dependent proliferation may shed light on the pathways that lead to the development of estrogen receptor-positive breast tumors. Estrogen regulates cyclin D1 expression and proliferation in breast cancer cells expressing estrogen receptor alpha (ER). However, function of ER is not sufficient for estrogen-dependent cyclin D1 expression and proliferation. Thus, genetic changes occurring during breast cancer development may allow the estrogen receptor to regulate cell cycle progression by inducing cyclin D1 levels.

In this study we tested the role of AIB1 in estrogen-dependent cyclin D1 expression. AIB1, a steroid receptor coactivator, was isolated as a gene that is amplified in breast cancer. We hypothesized that during breast cancer development overexpression of AIB1 in mammary epithelial cells confers estrogen-dependent cyclin D1 expression and proliferation.

To assess the role of AIB1, we overexpressed AIB1 in HaCaT-ER cells. HaCaT-ER cells express functional ER but cannot induce cyclin D1 expression or proliferation in response to estrogen. Increases in the levels of AIB1 expression led to proportional increases in estrogen-dependent cyclin D1 expression. To further characterize the regions of the cyclin D1 promoter required for AIB1-dependent cyclin D1 induction, we generated several deletion mutants for the cyclin D1 promoter. Deletion of AP-1 sequences abolished the ability of AIB1 to confer estrogen-sensitivity to the cyclin D1 promoter. In summary, high levels of AIB1 can enhance estrogen-dependent activation of the cyclin D1 promoter. This effect seems to be mediated through the AP-1 sequences in the cyclin D1 promoter. Thus, we conclude that overexpression of AIB1 allows productive interaction between the cyclin D1 promoter and ER. Blocking estrogen-dependent cyclin D1 expression may be a new way to prevent and/or treat estrogen receptor-positive breast cancer.

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THE KAR3 MOTOR PROTEIN IN YEAST MEIOSIS

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Understanding how cell-cycle checkpoints work is important to determine the defects in cancerous cells. This study set out to isolate checkpoint genes in meiosis using the kar3Δ meiotic arrest as a tool. This study was also used to characterize the meiotic arrest of kar3Δ mutants.

Genetics, cell biology, and molecular biology were used to pursue this goal.

KAR3 encodes a kinesin-like motor protein that has a wide variety of roles in the budding yeast Saccharomyces cerevisiae. Bypass studies have been done to discern the nature of the kar3Δ meiotic arrest and to find genes involved in checkpoint function that mediate the arrest. Checkpoints are important regulatory mechanisms used by cells to prevent aberrant cell cycle divisions such as those observed in cancerous cells. Data suggesting that the kar3Δ meiotic arrest is checkpoint mediated was generated. In addition, Cik1p and Vik1p are kinesin-associated proteins known to modulate the function of Kar3p in the microtubule dependent processes of karyogamy and mitosis. Experiments were performed to determine whether Cik1p and Vik1p are also important for the function of Kar3p during meiosis. The meiotic phenotypes of a cik1 mutant were found to be similar to those of kar3 mutants. Cells without Cik1p exhibit a meiotic defect in homologous recombination and synaptonemal complex formation. Most cik1 mutant cells, like kar3 mutants, arrest in meiotic prophase, however in cik1 mutants this arrest is less severe. These data are consistent with the model that Cik1p is necessary for some, but not all, of the roles of Kar3p in meiosis I. vik1 mutants sporulate at high levels, but have reduced spore viability. This loss in viability is partially attributable to vegetative chromosome loss. Cellular localization experiments reveal that Kar3p, Cik1p, and Vik1p are present throughout meiosis and are consistent with Cik1p and Vik1p having different meiotic roles. Lastly, chromosome dynamics were cytologically assayed in wildtype and kar3Δ cells throughout meiosis I. These experiments reveal that the kinetics of homologous chromosome pairing is altered in kar3Δ mutants.

This study has provided a good project for my research training, and has given us insight into the role of Kar3p in meiosis and checkpoints.

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THE RASSF1A TUMOR SUPPRESSOR INHIBITS CELL CYCLE PROGRESSION AND BLOCKS CYCLIN D1 ACCUMULATION

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The RASSF1A locus at 3p21.3 is epigenetically inactivated at high frequency in a variety of solid tumors. Expression of RASSF1A is sufficient to revert the tumorigenecity of human cancer cell lines. In an attempt to identify the mechanism by which RASSF1A tumor suppressor impacts growth regulation, we ectopically expressed RASSF1A in cancer-derived cell lines that had lost expression of endogenous RASSF1A and also into primary epithelial cell lines. We show here that RASSF1A can induce cell cycle arrest by engaging the Rb family cell cycle checkpoint. RASSF1A inhibits accumulation of native cyclin D1, and the RASSF1A-induced cell-cycle arrest can be relieved by ectopic expression of cyclin D1 or of other downstream activators of the G1/S- phase transition (cyclin A and E7). Regulation of cyclin D1 is responsive to native RASSF1A activity, because RNA interference-mediated downregulation of endogenous RASSF1A expression in human epithelial cells results in abnormal accumulation of cyclin D1 protein. Inhibition of cyclin D1 by RASSF1A occurs posttranscriptionally and is likely at the level of translational control. Rare alleles of RASSF1A, isolated from tumor cell lines, encode proteins that fail to block cyclin D1 accumulation and cell cycle progression. RASSF1A (A133S) is a consequence of a single nucleotide polymorphism. Because this variant shows defective antiproliferative activity in our assays, it is possible that individuals carrying this polymorphism may have increased risk for development of some types of neoplastic disease. Our results strongly suggest that RASSF1A is an important human tumor suppressor protein acting at the level of G1/S-phase cell cycle progression.

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IN VIVO ROLE OF D-TYPE CYCLINS IN DEVELOPMENT AND BREAST CANCER

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D-type cyclins are important in the G1 phase of the cell cycle. They physically interact with the cyclin-dependent kinases cdk4 and cdk6, as well as the retinoblastoma tumor suppressor gene product, pRB. While there is a large area of redundancy in D-type cyclin expression, individual knockout animals have illustrated that each one has specific functions in certain tissue compartments. For example, knockout mice lacking cyclin D1 develop hypoplastic mammary glands that fail to proliferate normally during pregnancy. This is likely due to the high expression pattern of cyclin D1 in this tissue. To examine the role of cyclin D1 in mammary tumorigenesis, we crossed cyclin D1 knockout and wild type animals with various strains of MMTV transgenic mice that were prone to developing breast cancers. These included MMTV-wnt-1, MMTV-myc, MMTV-ras, and MMTV-neu transgenic strains. The absence of cyclin D1 had no effect on the development of mammary tumors in MMTV-wnt-1 and MMTV-myc animals. MMTV-ras and MMTV-neu animals lacking cyclin D1 were completely protected from the development of tumors, indicating a critical role for cyclin D1 downstream of ras and neu signaling. Control cyclin D2 and D3 knockout animals were not protected from MMTV-ras driven oncogenesis, indicating a unique role for cyclin D1 in ras induced breast cancer. The question of whether this phenomenon is due to the specific expression patterns of the cyclins or an inherent difference in these cyclins is still a major question. This question will be addressed by the creation of a knockin animal that expresses cyclin D2 in the place of cyclin D1. Phenotypic studies of this strain should elucidate the in vivo role of the cyclin D1specificity as it pertains to normal mammary gland development and breast cancer.

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BREAST TUMOR KINETICS IN P53+/- AND RB+/- MICE TRANSGENIC FOR HUMAN CYCLIN E

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Deregulation of normal cell cycle control is a hallmark of tumorigenesis. One trait, elevated expression of cyclin E, may be of both prognostic value and play a key role early in neoplasm formation, at least in a large subset of spontaneous breast tumors.

In many breast tumors there is elevated expression of cyclin E. In transgenic mice that express human cyclin E, or a hyperstable form (T380A), in the mammary epithelia, 10-25% develop mammary tumors. This suggests that over expression of cyclin E is causative of tumorigenesis. To test if cyclin E causes genomic instability in vivo, we developed murine models that express either the wild-type or hyperstable mutant human cyclin E but are also heterozygous at the tumor suppressor loci Trp53 or Rb-1. We expected they would develop increased incidence of mammary tumors and at an earlier age.

Mammary tumorigenesis was not evident in any strain heterozygous at the RB-1 locus before morbidity from pituitary or thyroid lesions became apparent. However, in the strain carrying the hyperstable form of cyclin E and heterozygous at the Trp53 locus, mammary tumour penetrance was significantly enhanced (63%; 9/14). Furthermore, this was greater than possible additive incidence in p53 heterozygotes (8%; 4/50) or the transgenics alone (15%; 6/39) ($\chi 2 = 8.7$; $p<0.01$). Initial tumor analysis suggests that loss of the wild-type p53 is a feature common to all of the mammary tumours in this study. Mammary tumors from Cyclin E transgenic mice frequently maintained expression of the transgene, even in serial cell culture, suggesting that deregulated cyclin E expression may confer a significant growth advantage both in vivo and in vitro. Histologically, all tumors were high grade and locally invasive but without metastases while hyperplastic alveolar nodules were a frequent lesion observed in mice not bearing overt tumors. However, tumor latency was not decreased with most approximately correlating with the onset of reproductive senescence.

We interpret these results as the induction of genetic lesions leading to a preneoplastic state and eventually to mammary tumourigenesis. However, not unlike human sporadic cases, these tumors may also require the hormonal influence associated with reproductive senescence.

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MECHANISMS FOR REGULATION OF CYCLIN D1 EXPRESSION IN BREAST CANCER CELLS BY THE CYCLOPENTENONE PROSTAGLANDINS

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Evidence indicates that overexpression of cyclin D1 is an important event in malignant transformation of breast cancer cells. Therefore, cyclin D1 is a potential target for mechanistically-based chemoprevention/treatment of breast cancer. Treatment of MCF-7 breast cancer cells with the cyclopentenone prostaglandin 15d-PGJ2 results in rapid down-regulation of cyclin D1 protein expression and growth arrest in the G0/G1 phase of the cell cycle. The effect of 15d-PGJ2 on cyclin D1 is apparent within 30 to 60 min and maximal at 2 h after addition. 15d-PGJ2 is approximately 60 times more active than the related prostaglandin PGA2 in reducing cyclin D1 protein expression. 15d-PGJ2 also down-regulates the expression of cyclin D1 mRNA; however, this effect is delayed relative to the effect on cyclin D1 protein levels, suggesting that the regulation of cyclin D1 occurs at least partly at the level of translation or protein turnover. Treatment of MCF-7 cells with 15d-PGJ2 leads to a rapid increase in the phosphorylation of protein synthesis initiation factor eIF-2alpha and a shift of cyclin D1 mRNA from the polysome-associated to free mRNA fraction, indicating that 15d-PGJ2 inhibits the initiation of cyclin D1 mRNA translation. The selective rapid decrease in cyclin D1 protein accumulation is facilitated by its rapid turnover ($t_{1/2}$ = 34 min) following inhibition of cyclin D1 protein synthesis. Treatment of cells with 15d-PGJ2 results in strong induction of heat shock protein 70 (HSP70) gene expression, suggesting that 15d-PGJ2 might activate protein kinase R (PKR), an eIF-2alpha kinase shown previously to be responsive to agents that induce stress. Taken together, these results indicate that there are two mechanisms for regulation of cyclin D1 expression by 15d-PGJ2: rapid inhibition of translation of cyclin D1 followed by repression of cyclin D1 gene expression. Further studies are currently underway to completely elucidate the molecular mechanism for regulation of cyclin D1 translation and gene expression by 15d-PGJ2 and by the model compound 2-cyclopenten-1-one (cyclopentenone), which also down-regulates the expression of cyclin D1 and causes growth arrest of MCF-7 cells.

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A CANDIDATE TUMOR SUPPRESSOR THAT REGULATES UBIQUITIN-DEPENDENT TURNOVER OF CYCLIN E

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The development of breast cancer is associated with multiple genetic alterations that disrupt the regulatory mechanisms of the cell cycle and ultimately lead to uncontrolled proliferation. Key regulators of the eukaryotic cell cycle are the cyclin-dependent kinases (Cdks) which drive the major cell cycle transitions by phosphorylating a number of substrates. The activity of Cdks is tightly regulated by temporal association with their regulatory subunits, the cyclins. The association of the G1 cyclin, cyclin E, with Cdk2 is essential for entry of cells into S phase and the initiation of DNA replication. Cyclin E is synthesized periodically and accumulates to high levels at the G1-S transition followed by rapid destruction as cells enter S phase. We have found that ubiquitin-dependent degradation of cyclin E is controlled by a novel class of E3 ligases, termed SCF. SCF ubiquitin ligases are composed of a core complex of Skp1, Cul1 and Roc1 linked to a variable component known as F-box protein, which provides substrate specificity.

Recently, we have identified a novel human F-box protein, hCdc4, that specifically targets phosphorylated cyclin E for SCF-mediated ubiquitination. Deregulation of cyclin E is frequently observed in human cancers, and elevated cyclin E levels have been associated with poor patient prognosis in breast cancer. Furthermore, over-expression of cyclin E in transgenic mouse models leads to tumorigenesis, and constitutive expression of cyclin E causes genomic instability in cultured cells. Hyperaccumlation of cyclin E protein often occurs without a coordinate increase in cyclin E mRNA and mutations to the cyclin E gene are rare, suggesting the involvement of a post-transcriptional process. We have analyzed a panel of breast cancer derived cell lines and found that one line that exhibited high levels of cyclin E protein expressed an aberrant hCdc4 transcript. Mutational analysis of the hCDC4 locus revealed that both alleles were mutated resulting in the expression of a truncated hCdc4 protein that is no longer able to bind cyclin E. The loss of heterozygosity and the implication of elevated cyclin E in carcinogenesis suggest that hCdc4 may be a tumor suppressor. Our preliminary analysis of primary tumor samples supports this notion.

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CHK2 INTERACTS WITH POLO-LIKE KINASE 1

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The tumor suppressor protein kinase Chk2 plays an important role in mammalian DNA damage checkpoint pathway. It is a downstream target of Atm and Atr protein kinases, which phosphorylate Chk2 at T68 and at several additional sites within SQ/TQ cluster after DNA damage. This phosphorylation results in increased kinase activity of Chk2. Activated Chk2 regulates cell cycle transitions through phosphorylation of other proteins involved in DNA damage checkpoint like p53, cdc25A, cdc25C and BRCA1.

In attempt to identify new Chk2 substrates we exploited the homology between mammalian and yeast checkpoint pathways. Budding yeast checkpoint kinase RAD53, a homolog of Chk2, genetically interacts with CDC5. We have performed co-immunoprecipitation experiments which showed that Chk2 and Polo-like kinase 1(Plk1), a human homolog of CDC5, physically interact. In search for functional regulation we have co-transfected tagged versions of Plk1 and Chk2 and found that overexpression of Plk1 affects abundance of Chk2.

Plk1 is involved in regulation of mitosis and mitotic checkpoints. Interaction between Chk2, a bona fide DNA damage checkpoint protein, and Plk1 is a novel link between these two sets of checkpoint pathways.

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EVALUATING THE ROLE OF THE P53 HOMOLOGUE P63

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p63 is a recently identified member of the p53 family. The p63 gene encodes several splice variants which have trans-activating, apoptosis-inducing, and dominant-negative activities. Furthermore, the p63 proteins have significant homology to p53 (an amino acid sequence identity as high as 29% for the activation domain, 63% for the DNA binding domain, and 38% for the oligomerization domain) suggesting a possible role for p63 in modulating p53-mediated signaling. Moreover, studies have shown that p63 is amplified in squamous cell carcinoma of the head and neck. Subsequent studies showed p63 to be expressed in breast myoepithelial cells (basal epithelia of normal mammary epithelia) and certain subsets of neoplastic breast tissues. Because p63 has been implicated in the maintenance of the stem cell population in the basal layer of stratified epithelium, we believe that p63 may function as a dominant negative in suppressing p53 signaling in numerous epithelial tissues including breast. In support of this, using keratinocytes as a model system, we have found several splice variants of p63 RNA expressed in normal keratinocytes and tumor cells of the head and neck, but only one of these splice variants (ΔNp63a), which lacks a transactivation domain, was found to be expressed at the protein level. Several studies have suggested this role for p63 regulation of p53 signaling, but conclusive data demonstrating p63 binding to p53 consensus DNA sites in vivo, and negative regulation of p53 mediated transcription, has yet to be shown. To further explore this possibility, we are exploring Chromatin Immunoprecipitation (ChIP) assays to demonstrate p63 binding to p53 consensus DNA binding sites in vivo. We are also utilizing luciferase reporter assays and northern analysis to evaluate whether ΔNp63a can negatively regulate several different p53 target genes (through p53 consensus DNA binding sites) in epithelial cells. In addition, experiments are being performed to determine a mechanism by which ΔNp63a may function as a transcriptional repressor. Current work is now focused on evaluating the effect of p63 on p53 signaling, as well as identifying novel p63 regulated genes, in normal and tumor derived keratinocytes.

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UNDERSTANDING SUBSTRATE RECOGNITION BY CYCLIN-DEPENDENT KINASES

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Cyclin/Cdk activity is required for cell cycle progression but the key substrates for these enzymes remain unknown. Previous work from our laboratory had identified a cyclin-binding sequence, now known as a Cy motif, that is present in many Cdk substrates and inhibitors and is required for the association of those factors with cyclin/Cdk complexes. To gain a better understanding of how these Cy motifs contribute to substrate recognition by Cdks and to allow for the more effective identification of unknown substrates, we conducted a kinetic and mutational analysis of the Cy motifs from the replication factor Cdc6 and the Cdk inhibitor p21.

For the kinetic studies, we determined the contribution of the Cy motif to the catalytic efficiency of Cdks using a series of peptides derived from Cdc6. These substrate peptides contained a consensus SPXK phosphorylation site and either a wild-type or mutated Cy motif. We found that the elimination of the functional Cy motif in a substrate greatly increased the Km of the peptide ~100-fold demonstrating that an intact Cy motif plays a critical role in targeting these peptides for phosphorylation by cyclin-Cdk complexes.

For the mutational analysis, we have constructed a series of p21 mutants containing amino acid substitutions in the Cy motif and characterized their ability to inhibit Cdk activity. We show that although the positions occupied by the conserved arginine and leucine are most important for the inhibitory activity of p21N, a large number of mutations are well-tolerated in these positions. We also find that certain mutations in the Cy motif differentially affect the ability of the p21N mutant to inhibit different cyclin/Cdk complexes.

From these studies, we conclude that (1) Cy motifs play a critical role in substrate recognition by cyclin-dependent kinases, (2) there exists a large amount of degeneracy in terms of which amino acids are tolerated at a given position in the Cy motif, and (3) Cy motifs contribute to the specificity of cyclin/Cdk complexes by targeting cellular factors to specific cyclin/Cdk complexes. Together, this data provides the rationale for studies underway in our laboratory focusing on developing a new class of Cdk inhibitors that specifically disrupt the Cy motif-cyclin interaction.

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CHANGES IN GROWTH CONTROL ASSOCIATED WITH TELOMERASE INDUCTION IN CULTURED HUMAN MAMMARY EPITHELIAL CELLS

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The failures to arrest growth in response to TGFbeta and to repress telomerase activity are key derangements that distinguish cultured normal human mammary epithelial cells (HMEC) from breast cancer cells. Our recent observations indicate that activation of high levels of telomerase activity may itself cause derangements in growth control sufficient to cause resistance to TGFbeta-mediated growth arrest. The purpose of our current studies is to determine how telomerase expression affects TGFbeta control of cell cycle progression.

Finite lifespan HMEC, like all normal human epithelial cells, ultimately growth arrest in the presence of TGFbeta. In contrast, immortally transformed and tumor-derived HMEC lines, like most immortal human epithelial cells, may proliferate indefinitely in the presence of TGFbeta, although often at a slower rate. In our recent studies of HMEC immortal transformation induced by exposure to carcinogens or the breast cancer-associated oncogene, ZNF217, acquisition of endogenous telomerase activity was closely correlated with subsequent gradual acquisition of TGFbeta resistance. Introduction of hTERT, the catalytic subunit of telomerase, into finite lifespan telomerase(-) HMEC lacking expression of the cdk inhibitor $p16^{INK4A}$, indicated that hTERT is the limiting component of telomerase activity, and is sufficient to produce an indefinite lifespan in these cells. Additionally, hTERT induced resistance to TGFbeta-induced growth arrest. Induction of TGFbeta resistance required catalytically active hTERT capable of extending telomeres in vivo.

We hypothesize that high hTERT expression may alter the abundance, modification, and/or spatial arrangements of signaling molecules mediating TGFbeta-induced growth arrest. Our recent results suggest that alterations in the phosphoinositol-3-kinase (PI3K) signal transduction pathway are involved in hTERT-induced TGFbeta resistance. Treatment of TGFbeta resistant HMEC with the PI3K inhibitor, Ly294002, at a concentration that had little effect on proliferation by itself, partially reversed the TGFbeta resistance. In addition, we found that hTERT-transduced HMEC failed to completely growth arrest in response to blockage of EGF receptor signaling. Thus we conclude that the TGFbeta resistant growth observed in hTERT-transduced HMEC may be indicative of a more generic relaxation of cell growth requirements involving aberrant PI3K signaling.

The induction of TGFbeta resistance by hTERT in cultured HMEC, both through exogenous introduction and endogenous reactivation, may well model a key change occurring during human breast carcinogenesis in vivo. By exploring the common thread connecting telomerase expression and resistance to TGFbeta-induced growth arrest, it may be possible to unify two divergent areas of significance for breast cancer development and treatment, and to provide new targets for therapeutic intervention in breast carcinogenesis.

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